Stearylated Arginine-Rich Peptides: A New Class of Transfection Systems

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Membrane-permeable arginine-rich peptides, such as HIV-1 Tat-(48–60), HIV-1 Rev-(34–50), and flock house virus (FHV) coat-(35–49), have been shown to possess the ability to transfect COS-7 cells with luciferase-coding plasmid as efficiently as polyarginine (MW 5000–15 000) and polylysine (MW 9800). Not only these virus-derived cationic peptides but also oligoarginines of 4–16 residues were found to be able to transfect cells. In the case of the Tat, FHV, and octaarginine peptides, N-terminal stearylation of the peptides increases the transfection efficiency by approximately 100 times to reach the same order of magnitude as that of LipofectAMINE, one of the most efficient commercially available transfection agents. Also, a certain correlation was observed between the transfection efficiency of stearyl-$(Arg)_n$ peptides (stearyl-R_n: n = 4, 8, 12, 16) and the membrane permeability of the corresponding $(Arg)_n$ peptides (R_n).

INTRODUCTION

Arginine-rich peptides derived from human immunodeficiency virus (HIV)-1 Tat and Antennapedia homeodomain have been reported to translocate through cell membranes quite easily and to be utilized as carriers of macromolecules such as proteins and oligoDNAs by conjugating these basic peptides with the cargo macromolecules (*1*–*10*). Intracellular delivery of β-galactosidase (120 kDa) was achieved by the covalent conjugation with these carrier peptides (*4*). OligoDNAs up to ~30 bases were also introduced to cells with the help of these carrier peptides aiming at antisense or triple-helix therapy (*9*, *10*). Recently, we reported that not only the Tat-derived peptide but also various arginine-rich basic peptides derived from RNA-binding proteins, such as HIV-1 Rev and flock house virus (FHV) coat proteins, can penetrate through the cell membranes and bring exogenous proteins into the cells (*11*). The peptides composed of only arginine residues were also found to have the above-mentioned property (*11*, *12*). The fluorescence microscopic observation indicated that the peptide comprising eight residues of arginine translocated through cell membranes quite easily and accumulated in the nucleus. There was a certain dependence on the chain length for the cellular localization as well as the membrane permeability among these oligoarginine peptides. Quite interestingly, not only the smaller but also the larger the number of arginine residues became, the lower the membrane permeability and the less evident nuclear accumulation of the peptides became. In the case of the peptide comprising 16 arginine residues, the peptide was prominently observed on the membrane, and the accumulation in the nucleus was not significant (*11*). The results contradicted our expectation that, if only the positive charge was the driving force for the translocation, the efficiency should become higher as the peptide charge increased.

On the other hand, polyarginine and polylysine have been employed for the delivery of plasmid DNAs and antisense oligonucleotides into mammalian cells (*13*). In the delivery using the above membrane-permeable arginine-rich peptides, the cargo proteins or oligoDNAs were covalently attached to the arginine-rich carrier peptides and applied to the cells. In contrast, transfection of the plasmid DNA and the antisense nucleic acid using these polypeptides was usually conducted simply by mixing them and forming a peptide/nucleic acid complex. In such a case, peptides of high molecular weight (5000 or the higher) have often been employed with the hope of forming a tight and compact complex with plasmid DNAs (*14*). The transfection efficiency of small peptides of a distinctive structure, such as octaarginine, has been studied less extensively compared with other cationic polymers. In relationship to the potential of the arginine-rich peptides as a protein carrier, we were interested in the applicability of these "short" arginine-rich peptides to the transfection for plasmid DNA of a few kilobase pairs. In this report, the transfection efficiency of arginine-rich membrane-permeable peptides (Figure 1) was assessed for the first time on the basis of the luciferase assay, using a strategy of simply mixing the peptides with the plasmid DNA. The effect of peptide modification by hydrophobic moieties was also examined.

EXPERIMENTAL PROCEDURES

Materials. Fmoc-amino acid derivatives were purchased from Calbiochem-Novabiochem (Läufelfingen, Switzerland). Rink amide resin (TGS-RAM) was purchased from Shimadzu (Kyoto, Japan). Poly-L-lysine (MW 9800) and poly-L-arginine (MW 5000–15 000) were from Sigma (St. Louis, MO). LipofectAMINE and fetal bovine serum (FBS) were obtained from Life Technologies (Rockville, MD). Dulbecco's modified Eagle's medium (DMEM) was from Nissui Pharmaceutical (Tokyo, Ja-

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HIV-1 Tat-(48-60): NH_2-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Pro-Pro-Gln-$CONH_2$

HIV-1 Rev-(34-50): NH_2-Thr-Arg-Gln-Ala-Arg-Arg-Asn-Arg-Arg-Arg-Arg-Trp-Arg-Glu-Arg-Gln-Arg-$CONH_2$

FHV coat-(35-49): NH_2-Arg-Arg-Arg-Arg-Asn-Arg-Thr-Arg-Arg-Asn-Arg-Arg-Arg-Val-Arg-$CONH_2$

R_n (n=4, 8, 12, 16): NH_2-$(Arg)_n$-$CONH_2$

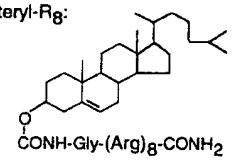
Stearyl-Tat: $CH_3(CH_2)_{16}$-CONH-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Pro-Pro-Gln-$CONH_2$

Stearyl-Rev: $CH_3(CH_2)_{16}$-CONH-Thr-Arg-Gln-Ala-Arg-Arg-Asn-Arg-Arg-Arg-Arg-Trp-Arg-Glu-Arg-Gln-Arg-$CONH_2$

Stearyl-FHV: $CH_3(CH_2)_{16}$-CONH-Arg-Arg-Arg-Arg-Asn-Arg-Thr-Arg-Arg-Asn-Arg-Arg-Arg-Val-Arg-$CONH_2$

Stearyl-R_n (n=4, 8, 12, 16): $CH_3(CH_2)_{16}$-CONH-$(Arg)_n$-$CONH_2$ Lauryl-R_8: $CH_3(CH_2)_{10}$-CONH-$(Arg)_8$-$CONH_2$

Cholesteryl-R_8:



Cholyl-R_8:

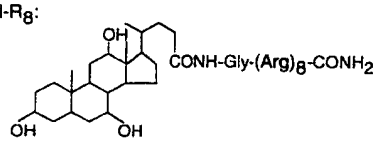


Figure 1. Structures of peptides used in this study.

pan). Plasmid DNA containing a firefly luciferase gene together with a simian virus 40 (SV40) promoter (PicaGene control vector2, PGV-C2) was from Toyo Ink (Tokyo, Japan). Other reagents were purchased from Aldrich (Milwaukee, MI) and Wako Chemical (Osaka, Japan).

Peptide Synthesis. All the peptides used in this report were synthesized by Fmoc-solid-phase peptide synthesis on Rink amide resin using a Shimadzu PSSM-8 synthesizer as reported (*15*). Stearyl-peptides were prepared by treatment of the corresponding peptide resins with stearic acid and diisopropylcarbodiimide in the presence of *N*-hydroxybenzotriazole followed by deprotection using trifluoroacetic acid (TFA)/ethanedithiol (EDT) (95:5). Lauryl-R_8 and cholyl-R_8 were similarly prepared using lauric acid and cholic acid in place of stearic acid, respectively. Cholesteryl-R_8 was prepared by treatment of the peptide resin with cholesteryl chloroformate in the presence of diisopropylethylamine followed by deprotection using TFA/EDT (95:5). All of the peptides were purified by reverse-phase HPLC. Fidelity of the products was ascertained by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS): HIV-1 Tat-(48−60), 1719.0/1719.1; HIV-1 Rev-(34−50), 2437.5/2437.8; FHV coat-(35−49), 2665.0/2664.5; R_4, 642.7/642.8; R_8, 1267.5/1267.5; R_{12}, 1891.5/1892.3; R_{16}, 2517.6/2517.0; stearyl-Tat, 1984.8/1985.2; stearyl-Rev, 2702.1/2702.8; stearyl-FHV, 2430.4/2430.6; stearyl-R_4, 909.2/909.2; stearyl-R_8, 1534.0/1533.9; stearyl-R_{12}, 2158.0/2158.7; stearyl-R_{16}, 2783.4/2783.5; stearyl-D-R_8, 1533.7/1533.9; lauryl-R_8, 1449.2/1449.9; cholesteryl-R_8, 1737.5/1737.3; cholyl-R_8, 1714.8/1715.2 [found/expected for $(M+H)^+$].

In Vitro Gene Transfection and Luciferase Assay. COS-7 cells, a simian kidney cell line transformed with SV40 (5×10^5 cells/well), were grown to just before confluence in a 24-well plate in DMEM containing 10% FBS under 5% CO_2 at 37 °C. Plasmid DNA coding a luciferase (PGV-C2, 5.2 kbp) (2.5 μg) in serum-free DMEM (25 μL) was added to the solution of a peptide or an N-terminal modified peptide in serum-free DMEM (25 μL), allowed to stand for 30 min, and diluted with serum-free DMEM (200 μL). The cells were washed 3 times with 500 μL of serum-free DMEM. The above peptide/DNA mixture was then gently added to the cells. After incubation for 12 h at 37 °C, DMEM (250 μL) containing 20% FBS was added. After 12 h, the medium was replaced with fresh DMEM (500 μL) containing 10% FBS. The cells were further incubated for 36 h. Harvest of cells and luciferase assay were performed following the protocol of a Pica Gene luminescence kit (Toyo Ink, Tokyo, Japan) as reported by Niidome et al. (*16*). The light units were analyzed by a BLR-201 luminescence reader (Aloka, Japan). The light unit values shown in the figures represent the specific luciferase activity (RLU/mg of protein) which is standardized for the total protein content of the cell lysate [10^6 RLU corresponds to 0.37 ng (6 fmol) of luciferase]. The measurement of gene transfer efficiency was performed in triplicate.

Transfection using LipofectAMINE was similarly conducted as stated above. Following the supplier's protocol, LipofectAMINE (2.5 μL) was employed for 2.5 μg of the plasmid DNA in place of oligoarginine in serum-free DMEM (25 μL).

Particle Size Determination. Particle size determination was carried out using a DLS-700 dynamic light scattering spectrophotometer equipped with a vertically polarized 5-mW He−Ne laser (633 nm) (Otsuka Electronics, Hirakata, Japan). Plasmid DNA (PGV-C2) (25 μg) in HEPES-buffered saline (HBS) (21 mM HEPES containing 135 mM NaCl, 5.0 mM KCl, and 0.76 mM Na_2HPO_4, pH 7.4) (250 μL) was mixed with the appropriate amount of a peptide or liposomes in HBS (250 μL) to give a cation: anion charge ratio of 2:1 and subjected to size determination.

Ethidium Bromide Displacement Assay (*17*). To a solution of plasmid DNA (PGV-C2) (3.5 μg) in HBS (600 μL) was added ethidium bromide (EtBr) (21 ng) in HBS (0.4 μL) and vortexed. An appropriate amount of stearyl-R_8 in HBS (2 μL) was added to the solution to give the desired cation:anion charge ratio. The fluorescence from the intercalated EtBr was monitored using a Hitachi fluorescence spectrophotometer F-3010 by the excitation and emission wavelengths at 510 and 595 nm, respectively.

MTT Assay (*18*). Cytotoxicity on transfection using stearyl-R_8 and LipofectAMINE was evaluated by MTT assay. COS-7 cells were placed in a 96-well plate (1×10^4 cells/well) in serum-free DMEM (50 μL) in the presence of the plasmid DNA (PGV-C2) (0.5 μg) and

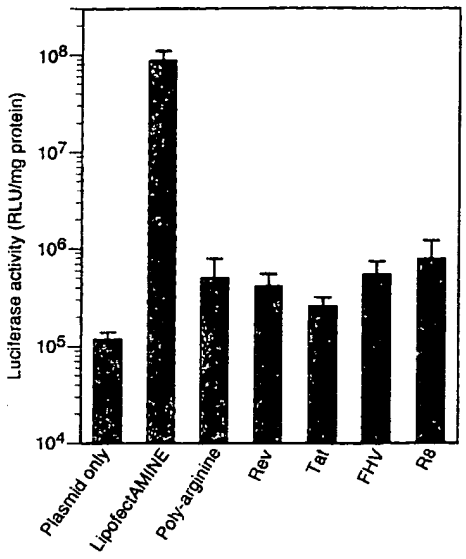


Figure 2. In vitro luciferase activity for PGV-C2 plasmid DNA after transfection of COS-7 cells using membrane-permeable arginine-rich peptides. Peptides were mixed with plasmid DNA at a cation:anion charge ratio of 2:1, where 15 nmol (as cation charge) of each peptide was used for 2.5 μg of plasmid DNA, respectively. Each bar represents the mean and standard deviation of three determinations.

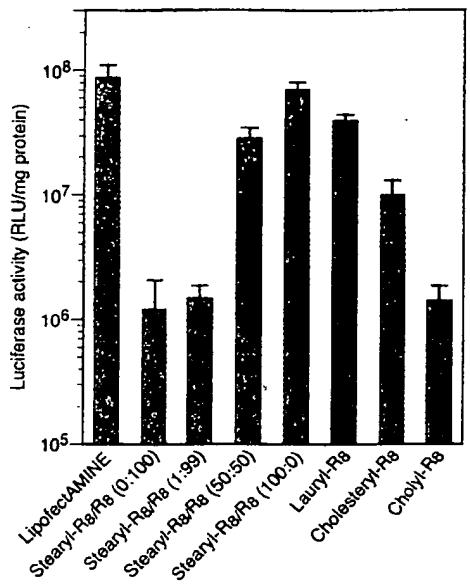


Figure 3. Effect of N-terminal modification of R_8 peptide on transfection efficiency. Peptides were mixed with plasmid DNA at a cation:anion charge ratio of 2:1, where 15 nmol (as cation charge) of each peptide was used for 2.5 μg of plasmid DNA, respectively. Each bar represents the mean and standard deviation of three determinations.

stearyl-R_8 (0.6 μg) [or LipofectAMINE (0.5 μL)] and incubated at 37 °C under 5% CO_2 for 12 h. DMEM containing 20% FBS (50 μL) was then added, and incubation was continued for another 12 h. The medium was removed, and the cells were treated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (50 μg) in DMEM containing 10% FBS (100 μL) for 4 h. Cell viability was expressed as the ratio of the A_{570} of cells treated with a transfection agent together with plasmid to that of the control samples.

RESULTS

Transfection Efficiency of Membrane-Permeable Arginine-Rich Peptides. The efficiency of HIV-1 Tat-(48−60), HIV-1 Rev-(34−50), FHV coat-(35−49), and octaarginine (R_8) in the transfection of COS-7 cells was examined using a plasmid DNA containing a firefly luciferase reporter gene (Figure 2). All of the peptides have already been proved to be highly membrane-permeable. As references, the transfection efficiency of conventional transfection agents, polylysine (molecular weight ~9800) and polyarginine (molecular weight 5000−15 000), was examined. In these experiments, a peptide and the plasmid were mixed in a cation:anion charge ratio of 2:1. Comparison of the transfection efficiency with that of LipofectAMINE, which is often employed as one of the most efficient cationic liposomes for transfection, was also conducted. The dose of LipofectAMINE for the plasmid DNA was selected following the manufacturer's protocol. The cation:anion charge ratio in this case was calculated to be 1.6:1. After mixing the plasmid with a peptide or LipofectAMINE, the mixture was applied to COS-7 cells, a simian kidney cell line transformed with SV40. Transfection was conducted for 12 h in the absence of serum, and the cells were incubated for 48 h in the presence of serum. Luciferase activity from the cells transfected with the peptides corresponding to HIV-1 Tat-(48−60), HIV-1 Rev-(34−50), and FHV coat-(35−49) was found to be comparable with that from the cells transfected with polyarginine. Transfection efficiency of the R_8 peptide was also determined to be comparable to those for the above peptides. Therefore, the arginine-rich peptides as short as ~10 residues have the ability to transfect the cells in almost the same efficiency as those for polylysine and polyarginine. In another words, a long peptide chain length is not necessarily needed for cationic peptide-mediated transfection. However, the transfection efficiency of these peptides was almost 2 orders of magnitude lower than that of LipofectAMINE.

Effect of N-Terminal Modification of R_8. To improve the transfection efficiency, efforts have been focused on the compaction of DNA-carrier complexes, with the hope of facilitating the cellular uptake of DNA through endocytosis or pinocytosis. For example, the application of polylysine bearing cysteine cross-linking (*19, 20*), branched cationic peptides (*17*), cationic polymers (*21*), and cationic dendrimers (*22*) has been reported. On the other hand, Nishikawa et al. successfully introduced hydrophobic cores such as aliphatic and cholesteryl moieties in the hydrophilic polysaccharides to obtain a complex of well-defined particle size (*23*). It has also been reported that the use of partially stearylated polylysine in the presence of low-density lipoprotein increased the transfection efficiency compared with the usage of polylysine (*24*). If arginine residues attach to the DNA phosphates, partial introduction of a hydrophobic moiety might result in the aggregation of these hydrophobic moieties to produce a compact plasmid/R_8 complex. Utilization of amino acid-based cationic lipid for the compaction of DNA has also been reported (*25, 26*). We thus prepared stearyloctaarginine (stearyl-R_8) as a model of the 'short' membrane-permeable arginyl peptides and examined the transfection efficiency of R_8 in the presence of stearyl-R_8 (Figure 3). The results were, however, not as intended. We did transfection using mixtures comprising stearyl-R_8:R_8 molar ratios of 0:100, 1:99, 50:50, and 100:0 (Figure 3). The transfection efficiency of these mixtures increased as the ratios of stearyl-R_8 increased. Maximum efficiency was obtained when 100% of stearyl-R_8 was employed for transfection.

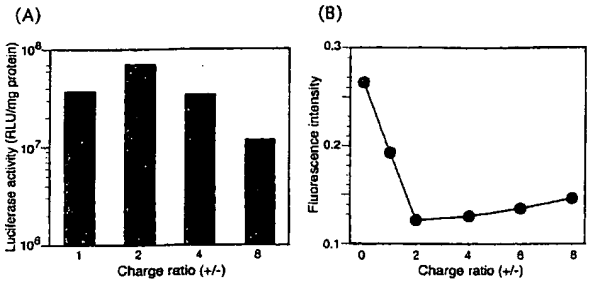


Figure 4. (A) Effect of cation:anion charge ratios on the luciferase activity from COS-7 cells transfected with plasmid DNA (PGV-C2, 2.5 μg) using stearyl-R_8. Each bar represents the mean of three determinations. The standard deviation in these cases was so small that the error bars do not appear on the figure. (B) Ethidium bromide (EtBr) displacement assay (*17*) for stearyl-R_8. Aliquots of stearyl-R_8 in HEPES-buffered saline (HBS) were added to a complex of plasmid DNA (PGV-C2) and EtBr in HBS. Decreases in fluorescence intensity, representing binding of the peptide to DNA and subsequent dissociation of EtBr, were recorded using excitation and emission at 510 and 595 nm, respectively.

In this case, transfection efficiency was improved by almost 2 orders of magnitude over the unmodified R_8; the efficiency became comparable to that of LipofectAMINE. The N-terminal stearylation of the R_8 peptide thus turned out to produce considerable improvement in the transfection efficiency.

The contribution of hydrophobic moieties to transfection efficiency has also been reported in other peptide carriers (*27*). Therefore, we prepared three more R_8 peptides bearing a hydrophobic moiety. These included lauryl-R_8, cholesteryl-R_8, and cholyl-R_8 peptides (Figure 1). Again, stearyl-R_8 gave the best transfection efficiency (Figure 3), followed by lauryl-R_8 and cholesteryl-R_8. A significant effect on modification was not recognized for cholyl-R_8.

Effect of Charge Ratio. The above results suggested that stearyl-R_8 is promising as a new type of transfection agent. To establish the peptide/DNA stoichiometry to achieve maximum transfection efficiency, the effect of the charge ratio of stearyl-R_8 and plasmid DNA was next examined. Transfection was carried out using different charge ratios of stearyl-R_8/DNA mixtures from 1:1 to 8:1. The highest luciferase activity was obtained from the cells treated with a cation:anion charge ratio of 2:1 (Figure 4A). The ethidium bromide displacement assay (*17*) showed a plateau in the fluorescence intensity at a charge ratio of 2:1 or greater, suggesting that stearyl-R_8 effectively complexed with DNA at a charge ratio of 2:1 and higher (Figure 4B).

Sequence Effect of the Arginyl Peptides. In our preceding paper on the membrane permeability of the arginine-rich peptides, we observed that a subtle difference in the number of arginine residues dramatically influenced the mode of membrane permeability and cellular localization of the peptides (*11*). A peptide comprising four arginine residues rarely crossed the membrane. In contrast, a highly efficient translocation and accumulation in the nucleus was observed for a peptide comprising six or eight arginine residues. However, as the number of arginine residues in the peptide further increased, membrane permeability again decreased. Internalization and nuclear localization of the peptide bearing 16 arginine residues were not significant; the peptide was observed mostly to stay on the membrane. A similar tendency was observed in the intracellular protein delivery using arginine-rich peptides as

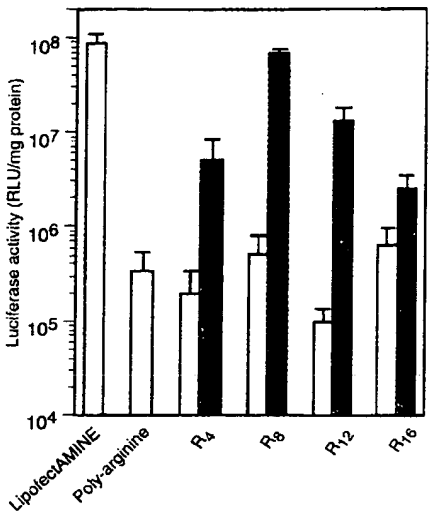


Figure 5. Comparison of transfection efficiency of stearyl-oligoarginine (stearyl-R_n) and oligoarginine (R_n) having a different number of arginine residues (n = 4, 8, 12, 16). Peptides (15 nmol as cation charge) were respectively mixed with plasmid DNA (PGV-C2, 2.5 μg) at a cation:anion charge ratio of 2:1 and used for transfection of COS-7 cells. Open bars indicate the transfection efficiency for R_n peptides. Closed bars indicate that for stearyl-R_n peptides.

a carrier molecule (*11*). Carbonic anhydrase (MW 29 000) covalently conjugated with a peptide comprising eight arginine residues was quite efficiently delivered into the cells. On the other hand, the protein conjugated with a peptide comprising 16 arginine residues was not able to translocate through the cell membrane as efficiently. Therefore, we examined whether a similar tendency was recognized in the transfection using stearyl-oligoarginine. In this experiment, DNA was simply mixed with stearyl-arginine peptides but not covalently cross-linked. We thus synthesized stearyl-oligoarginine of different chain lengths [stearyl-R_n (n = 4, 8, 12, 16)] (Figure 1). For comparison, oligoarginine peptides which lack the stearyl moiety [R_n (n = 4, 8, 12, 16)] were also prepared.

Without the stearyl moiety, slight differences were observed in transfection efficiency (Figure 5). However, the efficiency of these peptides was almost of the same order as that for polyarginine. On the other hand, considerable improvement in the transfection efficiency was observed by the N-terminal stearylation, especially in the case of stearyl-R_8 and stearyl-R_{12}. Almost 2 orders of magnitude increase in the transfection efficiency were obtained by stearylation of these peptides. Significant influence of the chain length on translocation efficiency was also observed. Stearyl-R_8 gave the best result in transfection efficiency. The smaller or the larger the number of arginine residues became, the less efficient was the transfection using the peptide. In the case of stearyl-R_{16}, improvement in translocation efficiency was not as obvious as in the case of other stearyl-R_n peptides. These tendencies were quite parallel to those observed in the above-mentioned internalization of the arginine peptides themselves and the peptide-mediated intracellular protein delivery (*11*). Transfection efficiency of the D-amino acid derivative of stearyl-R_8 (stearyl-D-R_8), where all the arginine residues were D-form, was 2.3 $\times$ 10^8 RLU/mg of protein, almost 3 times that of the L-form, stearyl-R_8. This result is also parallel to that observed in the quantification of internalized peptides where the (D-Arg)$_8$ peptide internalized more efficiently than the (Arg)$_8$ peptide (*12*).

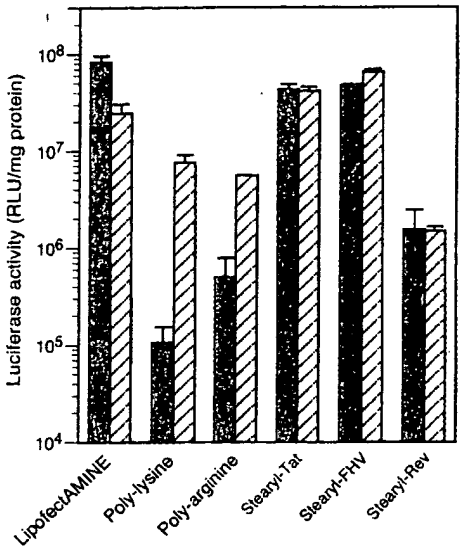


Figure 6. Transfection efficiency of stearylated arginine-rich membrane-permeable peptides and the effect of chloroquine. Peptides were mixed with plasmid DNA at a cation:anion charge ratio of 2:1, where 15 nmol (as cation charge) of each peptide was used for 2.5 μg of plasmid DNA, respectively. Cells were treated with 50 μM chloroquine during the transfection. Each bar represents the mean and standard deviation of three determinations. Closed bars indicate the efficiency in the absence of chloroquine. Hatched bars indicate that with chloroquine treatment.

The effect of stearylation on the transfection efficiency was also examined for the HIV-1 Tat-(48−60), HIV-1 Rev-(34−50), and FHV coat-(35−49) peptides (Figure 6). Two orders of magnitude of improvement were also observed by stearylation of the HIV-1 Tat-(48−60) and FHV coat-(35−49) peptides, and a transfection efficiency close to that of LipofectAMINE was achieved. Of interest is the result for the stearyl-Rev peptide. The ability for translocation as well as protein delivery was similarly observed for all three of these peptides. However, little improvement in transfection efficiency by stearylation was observed only for the Rev peptide.

Addition of chloroquine has often been reported to be effective in improving the transfection efficiency using polyarginine and other cationic peptides (*13*, *27*). A considerable increase was actually observed for the transfection efficiency using polylysine and polyarginine in the presence of chloroquine. For transfection using the stearyl-Tat and stearyl-FHV peptides, little improvement with the addition of chloroquine was observed (Figure 6). In the case of stearyl-R_n peptides (n = 4, 8, 12, 16), a significant effect of chloroquine on the transfection efficiency was not recognized (Figure 7).

Particle sizes of peptide/DNA complex were estimated using direct light scattering in HBS 3 min after mixing the peptides with DNA and then 30 min later. The latter incubation period is identical with that of the peptide/DNA mixture before the addition to cells (Table 1). Compared to polyarginine and LipofectAMINE, stearyl-R_8 formed a larger complex with DNA. The average particle size of the complex was almost 3 times larger than the lipofectAMINE/DNA complex 3 min after mixing, and 8 times larger after 30 min. The large standard deviation in the particle size also suggested that stearyl-R_8 formed complexes with DNA that easily aggregated into particles with a wide size distribution. While stearyl-Rev also formed large complexes with DNA, the average particle size and the standard deviation after 30 min were smaller than those for steary-R_8. The effect of R_8 on the compaction of DNA was not so large as that of polyarginine.

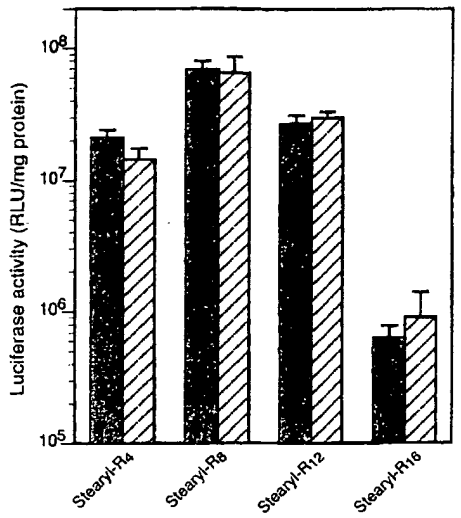


Figure 7. Effect of chloroquine on the transfection efficiency of stearyl-R_n peptides (n = 4, 8, 12, 16). Conditions are the same as mentioned in Figure 6. Closed bars indicate the efficiency in the absence of chloroquine (same as in Figure 5). Hatched bars indicate that with chloroquine treatment.

Table 1. Particle Size Determination of Peptide−Plasmid Complexes Using Dynamic Light Scattering[a]

	particle size (nm)	
	3 min	30 min
stearyl-R_8	1437 ± 1273	5148 ± 3377
stearyl-Rev	2198 ± 769	3692 ± 1120
R_8	1667 ± 537	2917 ± 738
poly-arginine	317 ± 43	663 ± 341
LipofectAMINE	462 ± 92	752 ± 398

[a] Each peptide was mixed with plasmid DNA (PGV-C2) to give a cation:anion charge ratio of 2:1. The amount of LipofectAMINE mixed with the plasmid was adjusted following the manufacturer's protocol. The particle size of each complex was determined 3 min after mixing and again 30 min later. The mean and standard deviation of three determinants are shown.

Cytotoxicity of the administration was assessed by the MTT assay (*18*) after incubation of cells with transfection reagents in serum-free medium for 12 h followed by incubation in serum for another 12 h. These incubation periods correspond to that where the peptide/DNA mixture was actually exposed to the cells. The cells were then harvested and subjected to the assay (Figure 8). The cytotoxicity of stearyl-R_8, polyarginine, and LipofectAMINE in the presence of plasmid DNA was judged to be comparable.

DISCUSSION

In this paper, we have exemplified that membrane-permeable arginine-rich peptides, such as HIV-1 Tat-(48−60), HIV-1 Rev-(34−50), and FHV coat-(35−49), have the ability to transfect COS-7 cells with luciferase-coding plasmid as efficiently as polyarginine (MW 5000−15 000) and polylysine (MW 9800). Not only these virus-derived cationic peptides, but also R_n peptides (n = 4, 8, 12, 16) were found to be able to transfect cells. In the case of the Tat, FHV, and R_8 peptides, stearylation on the N-terminus of the peptides dramatically increased the efficiency of transfection to reach the order of

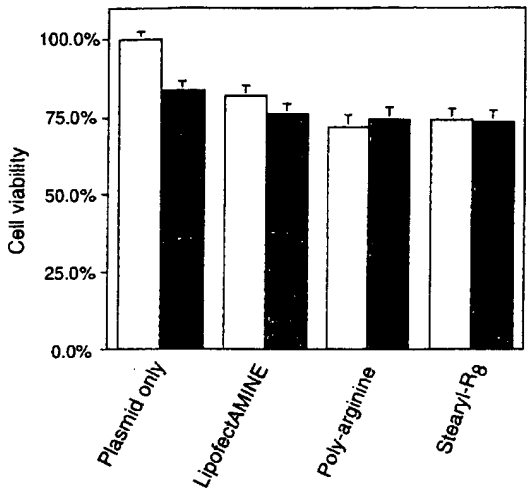


Figure 8. Cytotoxicity of the peptides and those complexed with the plasmid DNA. Cytotoxicity on transfection was evaluated by MTT assay (*18*). COS-7 cells were placed in a 96-well plate (1×10^4 cells/well) in serum-free DMEM and treated with a peptide or LipofectAMINE in the presence and absence of plasmid DNA (PGV-C2) for 12 h. An equal amount of DMEM containing 20% FBS was then added, and incubation was continued for another 12 h. The medium was removed, and the cells were subjected to MTT assay. Open bars, cell viability in the absence of plasmid DNA; closed bars, cell viability in the presence of plasmid DNA.

magnitude of that of LipofectAMINE, one of the most efficient commercially available transfection agents.

Polyarginine and polylysine of molecular weights more than 5000 have often been used for transfection. Presumably because of the availability of peptides, the transfection efficiency of 'short' arginine-rich peptides of distinctive molecular structure as used in this study has not been as extensively studied as those using cationic polypeptides and polymers. In relationship with the membrane permeability of these 'short' arginine-rich peptides, it is quite interesting that these peptides have the ability to deliver plasmid DNA into cells even without covalent conjugation with the plasmid.

N-terminal stearylation of the R_8 peptide showed a dramatic effect. Improvement of the transfection efficiency by almost 2 orders of magnitude was achieved. The effect of N-terminal modification by lauryl, cholesteryl, and cholyl moieties was studied. Modification by a straight chain lauryl moiety also gave about a 30-times increase in transfection efficiency. The cholyl moiety, which has an amphiphilic structure, contributed little to the improvement of transfection efficiency.

The average particle size of the stearyl-R_8/DNA complex 3 min after their mixing (1437 nm) was significantly larger than for the polyarginine/DNA complex (317 nm) and LipofectAMINE/DNA complex (462 nm). The average particle size of the stearyl-R_8/DNA complex increased faster than the other particles to exceed 5000 nm after 30 min. Because the 30-min incubation period was identical with that before transfection, it could be deduced that stearyl-R_8 forms a considerably larger complex than polyarginine and LipofectAMINE when added to the cells. Considering that stearyl-R_8 was added to plasmid DNA at a charge ratio of 2:1, it is expected that many stearyl moieties are exposed to the surface of the complex. One possible explanation for the high transfection efficiency of the stearyl-R_8/DNA complex could, therefore, be that the hydrophobic moiety contributes to absorbing of the complex on the membranes. A detergent-like effect could also be expected for stearyl-R_8 to destabilize the membranes to facilitate cellular uptake of the peptide/DNA complex.

One of the mechanisms for chloroquine to improve transfection efficiency has been attributed to destabilization of endosomal particles (*13*). Though a significant improvement was observed in the transfection efficiency for polyarginine and a slight decrease for LipofectAMINE with the addition of chloroquine, it did not produce a certain effect on the cellular uptake of the stearyl-R_n/DNA complex. The ineffectiveness of chloroquine on the transfection using the stearyl-R_8/DNA complex suggests that stearyl-R_8 would disrupt endosomes by itself. Also, the uptake mechanism of the stearyl-R_8/DNA complex by the cells may not be exactly the same as that of polyarginine and LipofectAMINE.

Dependence on the number of arginine residues for the transfection efficiency of stearyl-R_n peptides (n = 4, 8, 12, 16) correlated well with that observed for the internalization of the R_n peptides. Superiority of stearyl-D-R_8 over the L-peptide (stearyl-R_8) in transfection efficiency also coincided with that of the (D-Arg)$_8$ peptide over the (Arg)$_8$ peptide in membrane permeability reported by Wender et al. (*12*). The R_8 peptides showed the highest degree of nuclear localization among the R_n peptides in our previous observations (*11*). It would be quite interesting if the tendency in nuclear localization of R_n peptides could have something to do with that in transfection efficiency of the corresponding stearyl peptides. The Tat and FHV peptides also accumulate in the nucleus (*2, 11*), and the corresponding stearyl peptides gave high efficiency of transfection. Inefficiency of stearyl-Rev for transfection may have some relation to the peptide conformation. Among the arginine-rich peptides examined in this study, only Rev peptide takes on a helical structure (*11*). As a result, stearyl-Rev may form a complex with DNA in a different manner from those in the case of other stearyl peptides.

Taken together, the combination of the stearyl moiety and membrane-permeable basic peptides would provide a novel framework for the development of an efficient transfection system. Among other advantages expected for the stearyl-arginine peptides, low immunogenicity can be pointed out. Arginyl peptides delivered into the cells are expected to be easily degraded by cellular trypsin-like proteases as we observed in the translocation of arginine-rich peptides (*11*). Also, in accordance with degradation or dissociation of stearyl-arginine peptides in the cell, free plasmid DNA would be released to the cytosol followed by reaching the nucleus. It has been suggested that cationic polymers forming a very tight complex with DNA sometimes face difficulty in unwinding the complex in the cell, causing a reduction of transfection efficiency (*17*). COS-7 cells express the SV40 large T-antigen. This protein binds the SV40 promoter of the plasmid and may influence intracellular trafficking. However, the effect of the T-antigen on the delivery of the plasmid in the presence of stearyl-peptides is unclear at this stage.

Further study is necessary to understand the precise mechanism of transfection using stearyl-arginine-rich peptides. However, the above data as well as our previous report on peptide translocation (*11*) indicated that 'short' arginyl peptides are an abundant source of peptides having therapeutic potential. Further refinement of the peptide structure will result in more sophisticated molecules for intracellular gene delivery.

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